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Imaging SPR combined with stereoscopic 3D tracking to study barnacle cyprid-surface interactions

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Abstract

Barnacle larvae (cyprids) explore surfaces to identify suitable settlement sites. This process is selective, and cyprids respond to numerous surface cues. To better understand the settlement process, it is desirable to simultaneously monitor both the surface exploration behavior and any close interactions with the surface. Stereoscopic 3D tracking of the cyprids provides quantitative access to surface exploration and pre-settlement rituals. Imaging surface plasmon resonance (SPR) reveals any interactions with the surfaces, such as surface inspection during bipedal walking and deposition of temporary adhesives. We report on a combination of both techniques to bring together information on swimming behavior in the vicinity of the interface and physical interactions of the cyprid with the surface. The technical requirements for the setup are described, and we applied the setup to cyprids of *Balanus amphitrite*. Initial data shows the applicability of the combined setup to correlate exploration and touchdown events on surfaces with different chemical termination.

Introduction

Colonization of surfaces by marine organisms, commonly referred to as biofouling, happens as soon as surfaces are immersed into open waters. Adherent organisms on ship hulls, static structures, sensors and desalination plants are associated with huge costs for cleaning and maintenance (Townsin 2003). While the majority of coatings rely on the release of biocides, the most promising environmentally friendly antifouling strategies use weakening of adhesion strength (fouling release coatings), active interference with the adhesives, or inhibition of the adhesion process by rendering surfaces inhospitable (Callow and Callow 2011). As the latter approaches target the most important

step in biofouling - surface colonization - understanding exploration and selection of interfaces is of central relevance to guide coating development.

Barnacles are commercially important hard-fouling species (Aldred and Clare 2008). Heavily overgrown ship hulls cause an increase in fuel consumption by up to 60% (Schultz 2007). Surfaces become colonized by the larval stage of barnacles, cyprids, (Aldred and Clare 2008) and these cyprids show settlement preferences in so far as they select surfaces suitable for settlement and reject unsuitable ones (Aldred and Clare 2008). Video tracking allows observation and analysis of the motion of larvae and thus insight into their pre-settlement behavior (Lagersson and Høeg 2002, Marechal, Hellio et al. 2004, Prendergast, Zurn et al. 2008, Aldred, Li et al. 2010). Stereoscopic tracking adds the missing third dimension, required for calculation of parameters such as velocity and directedness, and enables a detailed analysis of cyprid distributions and swimming parameters as function of coating properties (Maleschlijski, Bauer et al. 2014, Maleschlijski, Bauer et al. 2015). Further, the surface exploratory behavior in direct contact with the substratum differs when different surface chemistries are applied (Chaw and Birch 2009). During this exploratory stage, cyprids "walk" across the surface using their two antennules. This interaction includes a temporary adhesive secreted at the terminus of the antennules, which enables temporary adhesion (Chaw and Birch 2009, Aldred, Li et al. 2010, Aldred, Høeg et al. 2013). As reported by Andersson et al. (Andersson et al. 2009), the application of imaging surface plasmon resonance microscopy (iSPR microscopy) allows quantitative analysis of the interaction of cyprids and their temporary adhesive with the surfaces. iSPR microscopy is an imaging optical transducer technique which measures refractive index changes of thin dielectric layers (de Bruijn et al. 1993), which can be translated into adsorbed amounts, or layer thicknesses. Using this method it is possible to quantify micro-scale bioadhesion events *in situ* in real time, and in particular to correlate these to variations in surface chemistry. For example, the occurrences of touchdown events and the amount of deposited organic material remaining after the interaction of the cyprids changes with the chemistry of the interfaces (Aldred, Ekblad et al. 2011). Combining conventional SPR methods with imaging SPR, the number of touchdowns by cyprids as well as the amount of the temporary adhesive material left behind on surfaces could be quantified.

As 3D tracking and imaging SPR provide complementary information, we have combined both techniques and here present the first results obtained with our new system. In this way dynamics of the swimming motion, detectable from 3D motility data, and of cyprid-surface interaction and exploration behavior can directly be correlated with iSPR. For these experiments, we used barnacle cypris larvae of the species *Balanus amphitrite*, which are considerably smaller than the *Semibalanus balanoides* larvae used in previous iSPR experiments (Andersson et al. 2009, Aldred et al. 2011), and whose footprints are more challenging to detect and record. Using two different self-assembled monolayers (SAMs) to vary the surface chemistry, we demonstrate that both surface interactions and bulk swimming patterns vary with the surface chemistry. The surfaces were chosen since cyprids are known to interact very weakly with oligo(ethylene glycol) chains and more strongly with carboxylic acid groups (Andersson, Ekblad et al. 2009, Aldred, Ekblad et al. 2011, Petrone, Aldred et al. 2015).

Methods

Preparation of surfaces

The glass coverslips exposing different surface chemistries were prepared from standard 0.17 mm glass coverslips (24 x 24 mm^2, VWR). The coverslips were cleaned in TL1 solution (25% NH_3, 30% H_2O_2 and water in 1:1:5 volume ratios, 10 min at 85 °C) before coating with a 10 nm adhesion-promoting titanium layer, followed by a 45 nm gold layer. The deposition was made in a custom-built electron-beam high-vacuum evaporation system. Two different self-assembled monolayers (SAMs) were used to vary the exposed surface chemistry, formed from either a methyl-terminated oligo(ethylene glycol)-functionalied alkylthiol, $HS(CH_2)_{11}CONH(C_2H_4O)_{11}CH_3$ (Polypure, Norway) (mPEG), or 11-mercaptoundecanoic acid, $HS(CH_2)_{10}CO_2H$ (Aldrich) (MUDA). The gold-coated coverslips were TL1-cleaned before incubation for at least 24 h in 50 µM thiol solutions in ethanol. The MUDA incubation solution was adjusted to pH 2 with HCl before use. Before use, the coverslips were sonicated for 2 mins in ethanol, rinsed and blown dry with nitrogen. Ellipsometric thickness measurements, sessile-droplet contact angle measurements and Fourier-transform infrared reflection-absorption measurements were carried out on samples prepared in parallel, to verify the quality of the SAMs. These data were consistent with previously published results from our laboratory, for both surface types (Petrone, Di Fino et al. 2011, Nugraha, Finlay et al. 2015).

Culture of cyprids

The experiments were performed with cyprids of the species *Balanus amphitrite* (= *Amphibalanus amphitrite*), which were cultured at Newcastle University, UK following established protocols (Imbesi et al. 2012). The cyprids were shipped in a cool-box overnight to Linköping University, Sweden where the experiments were done the following day. The age of the cyprids at the time of recording was 3-4 days. All experiments were performed in filtered (0.22 µm pore size) and sterilized (autoclaved) natural seawater. During the combined iSPR/stereoscopy experiments, approximately 20 swimming cyprids were kept in the sample container. Inactive or trapped cyprids were replaced, so as to maintain about 20 swimming ones throughout the experiment. Although clearly an artificial situation, this process improved the likelihood of cyprids leaving footprints in the limited iSPR field of view (see below) while reducing the difficulties associated with performing the stereoscopic analysis with a large number of cyprids. Exactly the same procedure was carried out on both surface types and the data are therefore comparable between treatments.

Cyprid settlement assays

Cyprid settlement assays were conducted in 24-well cell culture plates, as described previously by Di Fino et al. (Di Fino, Petrone et al. 2014). The plates were prepared as above, with the exception of the TL1 cleaning steps; instead, the plates were mounted in the evaporator immediately after breaking the sterile packaging, and filled with incubation solutions immediately after removal from the evaporation system. After sonication, rinsing and drying, the plates were kept in sealed bags under nitrogen until the settlement assays commenced. Settlement assays were conducted by adding 10 3-day old cyprids, in 2 mL of artificial seawater (Tropic Marin), to each of 12 wells per chemistry and incubating in darkness at 28° C for 24 h before counting the number of cyprids settled on the bottom or the walls of each well. The results are reported as means of the % of settled cyprids relative to the total number in the wells ± standard error (SE).

Imaging SPR setup and data analysis

The custom-built iSPR instrument, which has been described in detail previously (Andersson et al. 2009), is based on the Kretschmann configuration, see Figure 1A for a schematic illustration. A gold-coated glass coverslip is optically coupled to a BK7-glass prism (Melles Griot) with refractive index-matching oil (Cargille-Sacher Laboratories Inc.). White light from a 250 W tungsten lamp was monochromated, collimated, p-polarized and directed to the prism at 70° angle of incidence. The specularly reflected light from the prism was measured with a CCD camera (Retiga EXi, Qimaging Corp., monochromatic 12 bit, 1 Mpixel, no IR filter), using a 10x telecentric lens (Sill Correctal T). At this angle of incidence, the imaged area of the surface was approximately 900 x 2200 μm^2. Note that due to the oblique viewing angle, the useful field of view in the vertical direction (parallel to the plane of incidence) is less than the imaged area, since the the depth of field of the lens is much smaller than the projected length of the imaged area on the optical axis. Acquired images are normalized with respect to the reflection of s-polarized light. Continuous monitoring of cyprid interaction with the surfaces were made in intensity mode, keeping the wavelength fixed at at 665 nm, and monitoring intensity changes over time by capturing SPR images at a rate of 4 Hz using p-polarized light. An image acquired before the addition of cyprids is used as a reference, and is subtracted from subsequent images. For quantification of surface deposits, the changes in SPR wavelengths over the surface were determined by comparing images acquired before addition of cyprids, and after cyprid exploration of the surface. The wavelength was scanned from 600 to 700 nm, acquiring images at a separation of ca. 1 nm. From the resulting image stack, the SPR wavelength was determined by finding the reflectance minimum for each image pixel. The resulting SPR wavelengths from before and after the experiments were then compared, and the shift calculated for each point in the image.

Stereoscopy system

The stereoscopy setup consisted of two synchronized consumer camcorders as described recently (Maleschlijski et al. 2012). The cameras imaged the surface of interest from the top at a relative angle to each other of ca. 80° and a distance to the water surface of ca. 40 cm. A light source with a low intensity illuminated the scene from above (see Figure 1a). After the motions were recorded, a semi-automatic tracking algorithm was applied to extract trajectories from the videos. Therefore, the 2-D coordinates were recorded in both camera perspectives and merged into 3-D coordinates for each object of interest. To apply the epipolar transformation and to allow the extraction of quantitative information from the data points, both cameras were calibrated using a calibration object (see Figure 1a), following the procedure described in Maleschlijski et al. (Maleschlijski et al. 2012). The calibration object consisted of a precisely machined aluminum structure with rectangular posts of different height. z-values represent the distance to the surface, while the x and y values denoted the position in the surface plane. Connecting all 3-D coordinates of a single object of interest in time, produces a swimming trajectory from which quantitative parameters can be calculated.

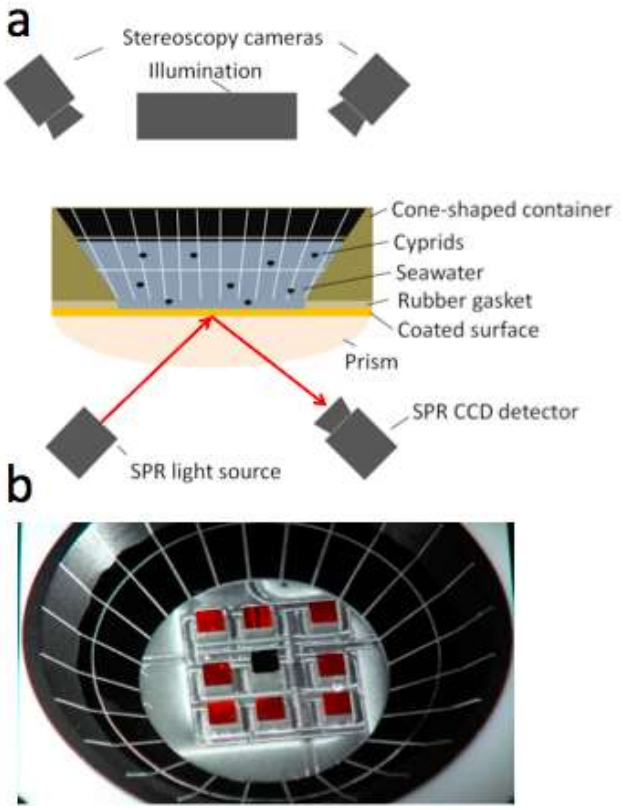

Figure 1: a) Schematic drawing of the setup. b) The cone-shaped container with coordinate grid, here shown with a calibration object at the bottom of the container. The container was designed to allow maximal field of view for stereoscopy, and at the same time easy exchange of the calibration object.

Results and discussion

Settlement of barnacle cyprids

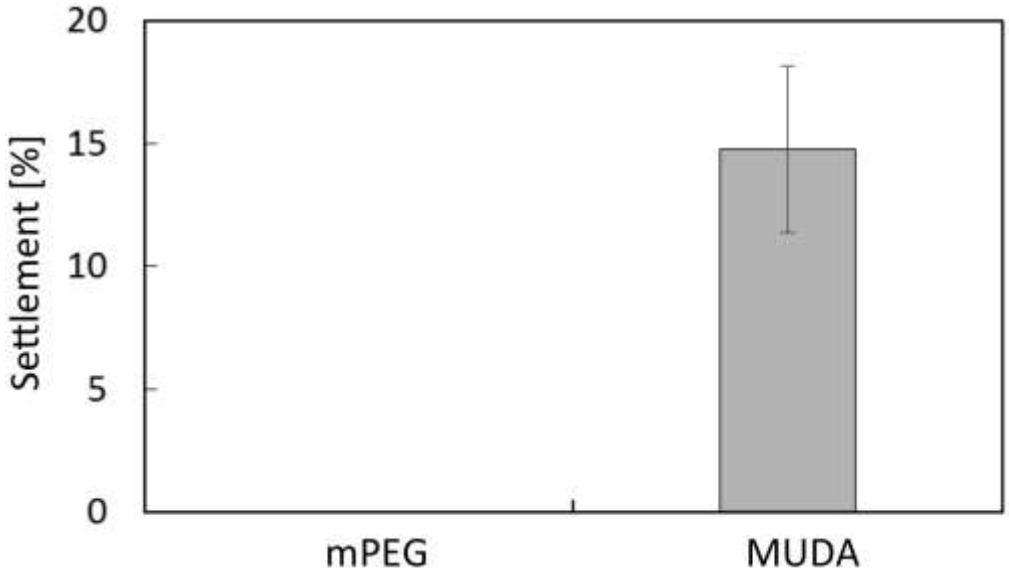

Figure 2: Cyprid settlement data after 24 h for the two surface chemistries used in the iSPR studies. Error bars represent 2 x standard error.

The expected differences in settlement preferences of *B. amphitrite* cyprids onto MUDA and mPEG surfaces were confirmed by a settlement test, where cyprids were allowed to explore the two different surface chemistries over a period of 24 h. The resulting settlement is shown in Figure 2. Although only around 15%, 24 h settlement on the carboxylated surface was actually quite high for a

SAM (which generally stimulate low settlement, even with an inductive chemical termination) whereas no settlement whatsoever was observed on mPEG. These results are in agreement with previous observations of high settlement of *B. amphitrite* on MUDA (Petrone, Di Fino et al. 2011), and low interaction of barnacle cyprids with mPEG surfaces observed in previous iSPR experiments (Andersson et al. 2009, Aldred et al. 2011).

Exploration and interaction with mPEG coated surfaces

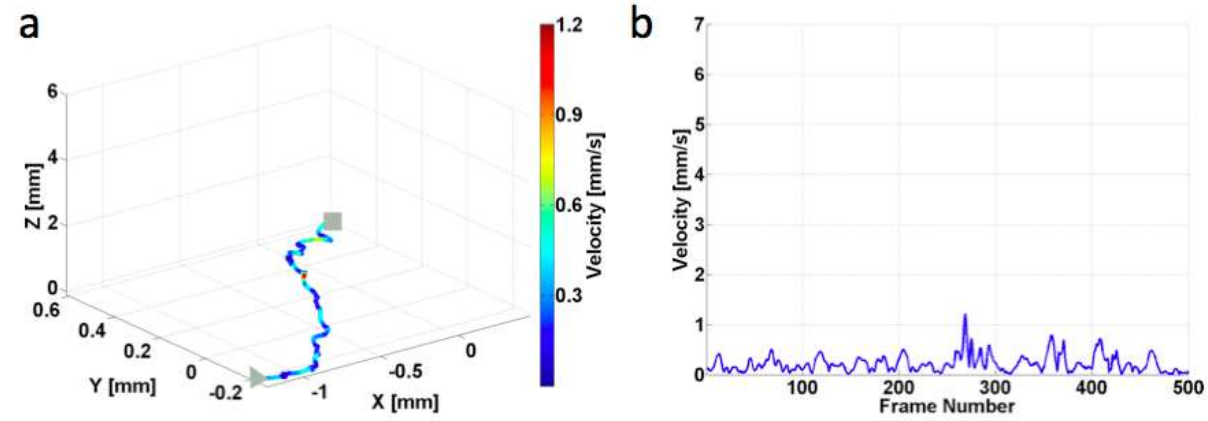

Figure 3: Cyprid approaching the mPEG surface. (a) A representative trajectory of a cyprid of *Balanus amphitrite* exploring the mPEG surface (the gray triangle and rectangular denote the beginning and end of the trajectory, respectively) and (b) the velocity profile of the trajectory. An exemplary touchdown event, detected by the iSPR during this segment of the trajectory, is shown in Figure 4.

Figure 3a shows a representative trajectory on the mPEG surface obtained with the stereoscopic part of the system. The cyprid swam close to the surface, did not change swimming patterns and exhibited low velocity. In addition, the velocity profile (Figure 3b) did not reveal any major changes and no distinct resting phases could be detected. On the contrary, a rather continuous and linear way of moving was observed. It seemed that the cyprid experienced the surface as non-attractive and the chemical cues did not trigger a pronounced close surface inspection behavior, but rather swimming close to the surface with occasional touchdowns (Matsumura et al. 2000; Lagersson & Høeg 2002; Chaw & Birch 2009; Pradhan et al. 2011).

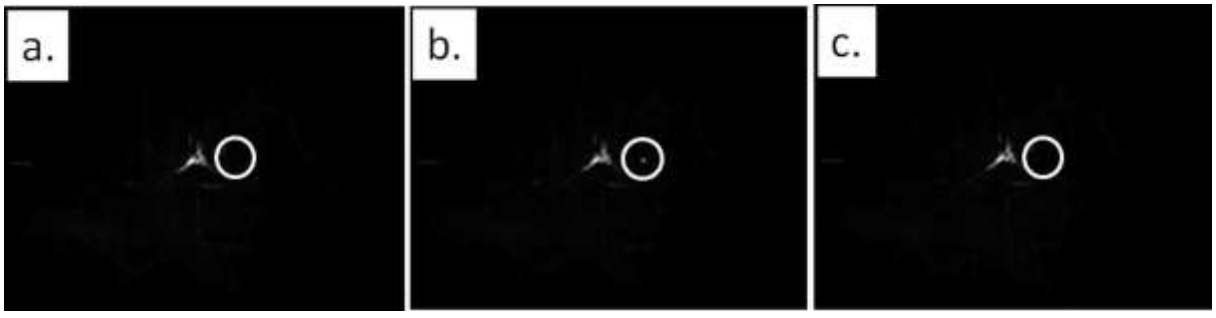

Figure 4: iSPR images demonstrating a touchdown event on an mPEG surface. The image sequence shows the occurrences of a touchdown event which was not observable (a) before the event, (b) occurred for a short period of time (marked with a white circle) and (c) disappeared after the event. The triangular spot in the center of the images is a reflection artefact.

In Figure 4, part of the iSPR signal corresponding to the representative trajectory is shown at three different time points around a touchdown event. The image series reveals that no iSPR signal was present before (Figure 4a) the touchdown. Interestingly, this event (Figure 1b) occurred for a very short period of time and then the signal disappeared (Figure 4c). The stereoscopic tracking information from Figure 3 reveals that during the observation time of the SPR the cyprid was exploring the surface and did not swim up in the water column. This means that the cyprid was

probing the surface and the occasional touchdowns became visble in SPR. After leaving the field of view, no organic deposit remained on the surface. Touchdown events without footprint deposition have already been demonstrated by Aldred et al. (Aldred et al. 2011) for non-attractive surfaces. Combining the findings from both systems it can be stated that the pre-settled behavior is dominated by a continuous motion close to the surface and complemented by few touchdown events which did not leave any footprints on the surface.

Exploration and interaction with carboxyl terminated surfaces

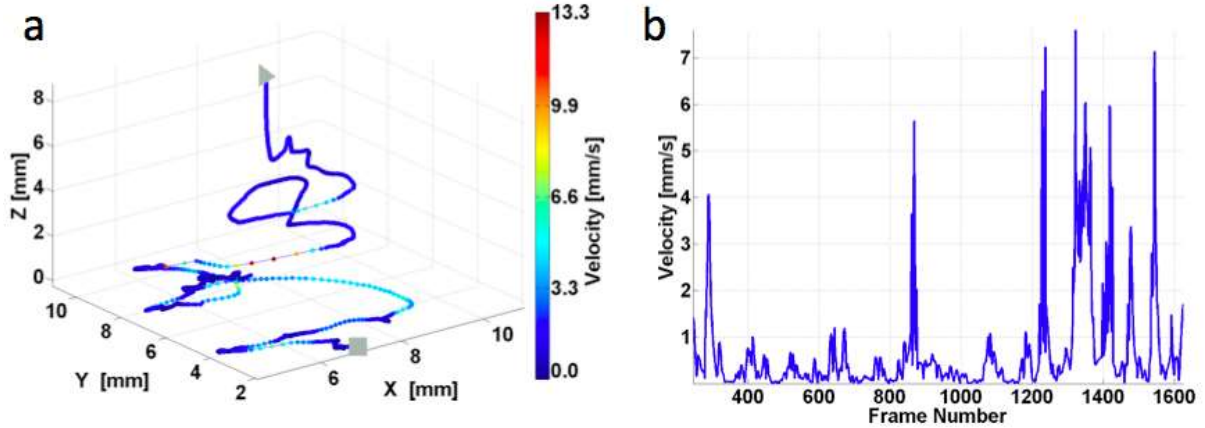

Figure 5: Cyprid exploring a MUDA surface. (a) A representative trajectory of a cyprid of *Balanus amphitrite* exploring the MUDA surface (the gray triangle and rectangular denote the beginning and end of the trajectory, respectively) and (b) the velocity profile of the trajectory. An exemplary touchdown event, detected by the iSPR during this segment of the trajectory, is shown in Figure 4.

The second tested surface, MUDA, is known to be attractive for settlement and a representative trajectory is shown in Figure 5a. The trajectory consisted of two parts - a swimming part in the water volume and swimming close to the surface with occasional surface contacts. The exploration part consisted of several walking segments (highly linear, with low velocity) which were connected by faster movements (up to ≈ 7 mm/s), shown in the velocity profile (Figure 5b). Especially the motion close to the surface contained a higher number of sharp turns as compared to the relatively smooth motion close to the mPEG surface.

SPR imaging of touchdown events on the carboxyl-terminated surfaces

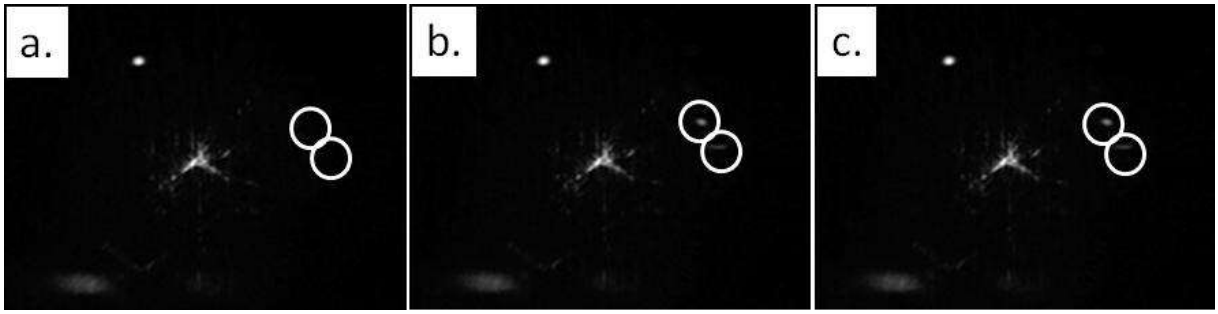

Figure 6: iSPR images demonstrating a touchdown event on a MUDA surface. The image sequence shows the occurrences of the touchdown events which were not observable (a) before the event, (b) occurred for a short period of time (marked with a white circle) and (c) remained there after initial touchdown, leaving an organic material behind.

A part of the iSPR signal obtained on the MUDA surface is shown in Figure 6. As can be seen in Figure 6b, the cyprids made two touchdowns and left an organic deposit behind (Figure 6c). It has been speculated in the literature whether the reason for leaving a high amount of footprints on the

negatively charged surface is related to the charge of the deposited material (Aldred et al. 2011) but this is still not fully understood. Combining the observations from both systems it could be concluded that the cyprids exhibited sharper turns and a less smooth behavior on the attractive MUDA SAMs. They swam in the water volume, engaged in surface exploratory behaviour, left footprints behind and eventually settled in larger numbers on these surfaces.

The deposits left on the MUDA surface were quantified by determining the shift in SPR wavelength caused by the deposit. No quantifiable deposits were found on the mPEG surfaces, while six investigated areas from three MUDA surfaces had in total 26 distinct footprints. The resulting wavelength shifts ranged from 6 to 10 nm, see Figure 7 for an example. Following the procedure by (Andersson, Ekblad et al. 2009), we used an approximate refractive index of 1.45 for protein deposits and an instrumental wavelenth shift of approximately 2 nm for every nanometer of deposited material (Andersson, Ekblad et al. 2009), to give a footprint thicknesses of 3-5 nm. This is slightly thinner than *B. amphitrite* footprint deposit thicknesses from AFM measurements reported in the literature, but it has also been shown that these quantities vary with surface chemistries and properties. E.g. Phang et al. reported a mean footprint thickness of 6-8 nm on surfaces with different chemical termination (Phang, Chaw et al. 2009) while Guo et al. observed even higher values (10-20 nm) on surfaces with different wettability (Guo, Puniredd et al. 2014).

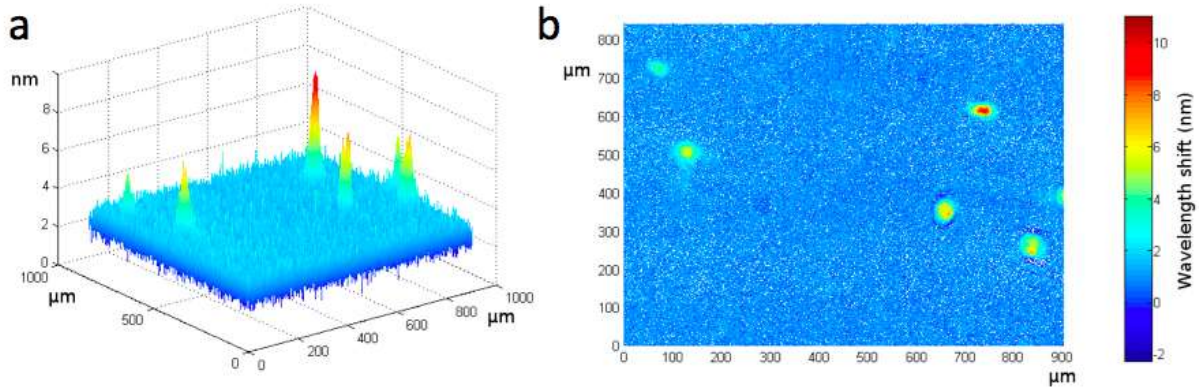

Figure 7. Resulting SPR wavelength shifts for footprints left on a MUDA surface. a) and b) show the same area, covering approximately 900 x 850 µm.

Summary and conclusion

We presented the combination of imaging surface plasmon resonance and video stereoscopy to understand interaction of barnacle larvae with surfaces. This novel system can be used to gain better knowledge of the swimming, exploratory and inspection behaviors of barnacle cyprids as well as the process of adhesive deposition. The advantage is that this can be observed simultaneously and the information is stored for post-processing. Using two surfaces with different chemical terminations - a highly hydrated mPEG and a negatively charged MUDA surface, we demonstrated that behavior and footprint deposition can be measured and correlated with settling probability. On mPEG, the cyprids showed relatively smooth swimming patterns and exhibited low velocities combined with fewer surface contacts, which did not leave any organic material deposited on the substrate. On the contrary, on the MUDA surface the larvae demonstrated patterns characteristic for surface exploration, with several direction changes and different velocities. Interestingly, on this surface more touchdown events occurred and organic material (`footprints') was left behind. These observations still remain to be confirmed statistically, but they already demonstrate that with this system effects can be revealed which remain covered for each of the techniques separately (Aldred et al. 2011; Maleschlijski, Bauer, Di Fino, et al. 2014; Maleschlijski, Bauer, Aldred, et al. 2014).

A severe limitation, originating from the nature of the iSPR technique, is the small size of the detected field or field of view. This highly limits the observable area of the iSPR and, thus, also of the stereoscopic system. A valid direction for improvement of the setup could be an automated stage for the imaging arm of the iSPR microscope, following the cyprids with help of real-time tracking and positioning of the imaging optics. Such an automated setup would allow to image cyprids during the whole cycle of exploration and surface inspection in the whole field of view, thus extending the knowledge of this highly dynamic process.

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